DIFFERENCES OF CHEMICAL STRUCTURES OF *PSEUDOMONAS AERUGINOSA* LIPOPOLYSACCHARIDE ESSENTIAL FOR ADJUVANTICITY AND ANTITUMOR AND INTERFERON-INDUCING ACTIVITIES

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1. Introduction

Adjuvanticity, antitumor and interferon-inducing activities of a protein rich endotoxin isolated from an autolysate of *Pseudomonas aeruginosa* have been demonstrated [1–4]. It has been reported that both antitumor and interferon-inducing activities reside in the lipopolysaccharide portion of the protein–lipopolysaccharide complex [5]. Moreover, chemical structures of the lipopolysaccharide essential for antitumor and interferon-inducing activities were clarified [5].

Here the relationship between chemical structures of lipopolysaccharide as well as their derivatives and adjuvanticity were investigated, comparing the results with those of antitumor and interferon-inducing activities previously reported.

2. Materials and methods

The protein-rich endotoxin was prepared in our laboratory by the method in [3] and subjected to various chemical treatments as follows. The details of these preparation procedures and chemical natures of each samples were reported [5]. Lipopolysaccharide from the protein–lipopolysaccharide complex was isolated by the phenol/water procedure. Lipid A from the complex or from lipopolysaccharide derived from the complex was obtained with 2% acetic acid at 100°C, 2.5 h treatment, and solubilized with triethylamine or complexed with bovine serum albumin. Polysaccharide was obtained from the supernatant of the mild acid hydrolysis reaction mixture after extraction of lipoidic substances with chloroform. Oxidized lipopolysaccharide was obtained by treatment with 0.1 M $NaIO_4$ at 4°C in the dark for 4 days (pH 2.0). Succinylation and phthalylation were by the method in [6]. The release of ester-linked fatty acid was brought about by hydroxylaminolysis (2% NH_2OH in 4% NaOH in ethanol, 63°C, 3 min) as in [7]. Completely de-acylated lipopolysaccharide was obtained by the treatment with anhydrous hydrazine for 40 h at 100°C. Partial de-*O*-acylation was performed by alkaline solution (0.1 M NaOH in 99% ethanol) at 37°C.

The adjuvant effects of the protein–lipopolysaccharide complex and its derivatives were estimated by an increase of direct plaque forming cell (PFC) assay in the spleen of mouse using sheep red blood cells (SRBC) (Japan Bioproduct Co.) as an antigen. SRBC suspended in Alserver solution and stored at 5°C were washed twice with a M/15 phosphate-buffered physiological saline (pH 7.0) (PBS) just before use. Week 5–6 CDF_1 female mice (Laboratory of Experimental Animals, Institute of Medical Science, University of Tokyo) were used. In all experiments, each group consisted of 5–10 mice. Mice were intraperitoneally injected with a simultaneous dosage of 30 μg test sample and 0.2 ml 10% SRBC. Control mice were injected with the same volume of saline instead of the test sample. All the mice were sacrificed on day 4 after immunization.

The number of hemolysin producing cells of spleens were examined by the direct plaque forming cell assay (direct PFC) of Cunningham's method.

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Spleens were cut into thin slices and the cells were pushed. The cell suspension was passed through stainless mesh (no. 200). The cells were washed twice with 5% fetal calf serum (FCS) PBS and suspended with 10% FCS Eagle MEM medium. Numbers of viable cells were counted by crystal violet staining.

The spleen cell suspension (0.4 ml), 50% SRBC (0.05 ml) and complement (0.05 ml) (pooled guinea-pig sera absorbed with SRBC) were mixed well, and 0.1 ml mixture placed in a Cunningham's chamber (Takahashi Giken Co.) and incubated at 37°C for 1 h. The number of hemolytic plaques were counted with microscope.

3. Results and discussion

The results of adjuvant effect of protein–lipopolysaccharide complex and lipopolysaccharide are shown in table 1. The lipopolysaccharide derived from the complex by phenol/water extraction showed itself to be more potent in adjuvanticity than that of the complex, indicating that the protein moiety is not essential to the adjuvant effect of the complex. Then lipid A fractions were isolated from both the complex and lipopolysaccharide and solubilized. The adjuvant activity of the two lipid A samples was compared with the original materials. As shown in table 2, these lipid A fractions showed almost the same values in adjuvanticity as that of control material. Adjuvant effect of the polysaccharide (protein and lipid free) derived from the isolation procedure of lipid A from lipopolysaccharide by acid hydrolysis showed no adjuvant activity.

To examine whether polysaccharide portion of lipopolysaccharide has an influence on exhibiting

Table 1
Adjuvant effect of protein–lipopolysaccharide complex and lipopolysaccharide derived from the complex of *Pseudomonas aeruginosa* on anti-sheep red blood cell immune response

Sample	Response (PFC/spleen)		Adjuvant index
	SRBC alone	With sample	
Protein–lipopolysaccharide complex from autolysate of *Pseudomonas aeruginosa*	1.09 ± 0.13	1.59 ± 0.14	1.47
Lipopolysaccharide from above complex	1.98 ± 0.20	3.40 ± 0.25	1.71

Mice (week 5–6, female) were injected intraperitoneally with 30 μg of each derivative and 0.2 ml SRBC, simultaneously. PFC response is expressed in the number of (direct PFC mean ± SE) × 10^5 /spleen 4 days after challenge (arithmetic mean of the response of 5–7 mice for each group)

Table 2
Adjuvant effect of various samples of free lipid A and polysaccharide

Sample	Treatment	Response (PFC/spleen)		Adjuvant index
		SRBC alone	With sample	
Lipid A from:				
complex	$(Et)_3N$	1.26 ± 0.25	1.70 ± 0.18	1.34 n.d.
lipopolysaccharide from complex	Albumin	2.02 ± 0.09	3.25 ± 0.26	1.61 (1.81)
Polysaccharide	–	2.04 ± 0.26	1.67 ± 0.27	0.81 n.d.

Measurements were made on various samples of lipid A before and after treatment with triethylamine [$(Et)_3N$] or bovine serum albumin. Numbers in parentheses indicate adjuvant index of lipopolysaccharide from the complex as a control. See note for table 1

Table 3
Adjuvant effect of chemically modified lipopolysaccharide samples

Sample	Response (PFC/spleen)		Adjuvant index
	SRBC alone	With sample	
Lipopolysaccharide:			
oxidized with $NaIO_4$	2.26 ± 0.15	3.38 ± 0.14	1.50 (1.58)
succinylated	2.12 ± 0.18	2.63 ± 0.16	1.24 (1.65)
phthalylated	1.98 ± 0.24	3.49 ± 0.54	1.76 (1.75)

See notes for tables 1,2

adjuvant effect of lipopolysaccharide or not, we modified chemically the polysaccharide portion either by $NaIO_4$, succinic anhydride or phthalic anhydride. These treatments are believed to destroy or modify the polysaccharide portion of the lipopolysaccharide molecule. As shown in table 3, both of the phthalylated lipopolysaccharide and lipopolysaccharide oxidized with $NaIO_4$ preserved almost the same adjuvant activity, although the succinylated lipopolysaccharide was found less potent in adjuvanticity than lipopolysaccharide.

The above results indicate that only the lipid A portion is required for adjuvanticity. In further experiments (table 4) we studied the effect of deacylation of lipopolysaccharide on the activity. When the lipopolysaccharide obtained from the complex was treated by hydroxylamine or anhydrous hydrazine resulting in the removal of ester-linked fatty acids or all the fatty acids, the adjuvant effect markedly decreased in both cases. If the sample was treated by mild alkaline solution (0.1 M NaOH), however, about half of the fatty acids were present and the adjuvant activity remained.

Table 4
Adjuvant effect of deacylated samples of lipopolysaccharide

Deacylation treatment	Response (PFC/spleen)		Adjuvant index
	SRBC alone	With sample	
NH_2OH	1.98 ± 0.24	2.54 ± 0.19	1.28 (1.75)
N_2H_4	2.27 ± 0.09	2.52 ± 0.21	1.11 (1.67)
0.1 N NaOH in 99% EtOH	0.84 ± 0.18	1.7 ± 0.13	2.02 n.d.

Lipopolysaccharide from the protein–lipopolysaccharide complex which has been isolated from the autolysate of *P. aeruginosa* was deacylated in various ways before measurements were made. See notes for tables 1,2

From these results, the lipid A portion with an adequate amount of ester-linked fatty acids as well as amide-linked fatty acids is shown to be essential for demonstrating adjuvant activity.

In [5], it was shown that, for the inhibition of ascites tumor development, lipid A alone is not sufficient and such a saccharide as 3-deoxy-D-manno-octulosonic acid is necessary to link the structure of the lipid A portion. In contrast, the incomplete lipid A with amide-linked fatty acid alone is sufficient to induce interferon. Accordingly, biological activities such as adjuvanticity, antitumor and interferon-inducing ones of the lipopolysaccharide can be dissociated according to chemical structures of lipid A and its derivatives.

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